BINDING OF FLUORESCENT PROBES TO MICROSOMES WITH REGARD TO THE FUNCTION OF THE MONO-OXYGENASE COMPLEX

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1. Introduction

Fluorescent probes have been used as tools to investigate structural and functional relationships in biological membranes [1,2]. In studies on microsomes, fluorescent probes have been used to study the interaction of drugs, steroid hormones and metal ions with the membrane [3–5]. Probes have also been used to reveal structural changes in microsomal phospholipids [6–8]. 1-Anilinonaphtalene-8-sulphonate, which is known to bind to microsomal phospholipids [6] has been used to reveal the phase transition temperature range of the phospholipids which are important for the catalytic function of the microsomal mono-oxygenase complex [8].

Some reports have appeared where fluorescent probes have been used to study the reaction mechanism of microsomal oxidation of xenobiotics: 1,8-ANS has been found to decrease the binding of some substrates for microsomes [3,9] and to inhibit microsomal drug metabolism [7–9]. The interpretation of these findings requires, however, a closer understanding of the action mechanism of the probes, which is not yet known in detail.

This study has been carried out to obtain further information on the interaction of three probes, of different types, with the microsomal mono-oxygenase complex and on the mechanism by which they inhibit drug metabolism.

We found that of the three probes used 1,8-ANS, an anion, was the strongest and EtBr, a cation, the weakest inhibitor of the dealkylation reactions. The mechanism of inhibition by 1,8-ANS and to a lesser extent by PNA, a nonionic probe, was found to be interference in the electron transport from NADPH cytochrome *P*-450 reductase to cytochrome *P*-450.

Abbreviations: 1,8-ANS, 1-anilinonaphtalene-8-sulphonate; PNA, *N*-phenyl-1-naphtylamine; EtBr, ethidium bromide

2. Materials and methods

Microsomal fraction was separated from the livers of 3 male Wistar rats (250 ± 15 g). The rats were killed by a blow on the head and were bled by cutting the renal vessels, after which livers were immediately dissected and placed in ice-cold 0.25 M sucrose solution. Livers were then homogenized in 4 vol. 0.25 M sucrose solution with a Potter-Elvehjem-type glass–teflon homogenizer (370 rev/min; 5 pestle strokes). The homogenate was then spun at 10 000 × *g* for 20 min and the microsomes were separated from the supernatant by centrifuging at 105 000 × *g* for 60 min (Sorvall OTD-2). The microsomal fraction was resuspended in 0.15 M KCl. After harvesting by centrifuging (105 000 × *g* for 45 min) the washed microsomes, were finally suspended in 0.1 M Tris–HCl buffer, pH 7.4, to yield 1 ml/g liver wet wt containing 20.5 mg protein/ml.

Protein content of the microsomal suspension was determined by a modified biuret method [10,11]. The demethylation of *p*-nitroanisole by microsomal mono-oxygenase complex was determined as in [12] modified [13], and the de-ethylation of 7-ethoxycoumarin as in [14] modified [15]. The reactions were also determined by using cumene hydroperoxide (Fluka, Switzerland) as a substitute for molecular

oxygen and NADPH in the reaction mixture [16,17]. The activity of microsomal cytochrome *P*-450 reductase was determined by using a water soluble substrate 2,6-dichlorophenol indophenol (Merck) as in [18].

The mono-oxygenase complex catalyzed dealkylation reactions and the reduction rate of 2,6-dichlorophenol indophenol were determined also in the presence of fluorescent probes 1,8-ANS (Serva), PNA (Merck) and EtBr (Sigma) in the reaction mixture. Probe concentrations of 25 μM and 50 μM were used. Of the probes 1,8-ANS is anionic, EtBr cationic and PNA nonionic. To avoid possible spectral interferences in the determination of enzyme activities, an equimolar concentration of probes was added in control tubes after stopping the reaction. The same microsomal preparation was used for all of the enzyme determinations.

3. Results

The effect of the probes on the reduction of 2,6-dichlorophenol indophenol is shown in table 1. This reaction was inhibited slightly by 1,8-ANS whereas PNA and EtBr had no effect at the concentrations used.

The effect of the probes on NADPH-driven reactions of the mono-oxygenase complex is shown in table 2. It can be seen that 1,8-ANS is the most effective of the probes in lowering the rate of the reactions. PNA also decreased microsomal drug metabolism to some extent, although the effect was much weaker than that of 1,8-ANS (table 2). EtBr had little effect on the demethylation of *p*-nitroanisole and inhibited the de-ethylation of 7-ethoxycoumarin only slightly (table 2).

Table 1
Relative activity of the microsomal cytochrome *P*-450 reductase catalyzed reduction of 2,6-dichlorophenol indophenol in the presence of fluorescent probes in the reaction mixture

Control	1,8-ANS		PNA		EtBr	
	25 μM	50 μM	25 μM	50 μM	25 μM	50 μM
100	78	82	82	100	118	106

The specific activity for control was 42 nmol/min . mg prot. Mean values of 5 determinations are expressed

Table 2
Relative activities of microsomal, NADPH-driven dealkylation reactions in the presence of fluorescent probes in the reaction mixture

Control	1,8-ANS		PNA		EtBr	
	25 μM	50 μM	25 μM	50 μM	25 μM	50 μM
	Demethylation of *p*-nitroanisole					
100	33	21	72	63	89	102
	De-ethylation of 7-ethoxycoumarin					
100	27	20	75	65	85	70

The specific activities for controls were 0.65 nmol/min . mg prot. for *p*-nitroanisole *O*-demethylase and 0.73 nmol/min . mg prot. for 7-ethoxycoumarin de-ethylase. Mean values of 5 determinations are expressed

Table 3
Relative activities of cumene hydroperoxide-driven microsomal dealkylation reactions in the presence of fluorescent probes in the reaction mixture

Control	1,8-ANS		PNA		EtBr	
	25 μM	50 μM	25 μM	50 μM	25 μM	50 μM
	Demethylation of *p*-nitroanisole					
100	115	134	107	118	96	92
	De-ethylation of 7-ethoxycoumarin					
100	114	109	106	102	98	106

The specific activities for controls were 0.51 nmol/min . mg prot. for *p*-nitroanisole *O*-demethylase and 0.56 nmol/min . mg prot. for 7-ethoxycoumarin de-ethylase

From table 3 it can be seen that the cumene hydroperoxide-driven reactions were not inhibited by the fluorescent probes. Instead, the reactions seemed to be activated slightly by 1,8-ANS and PNA.

4. Discussion

A model for the microsomal mono-oxygenase complex has been proposed [19], in which cytochrome *P*-450 and cytochrome *P*-450 reductase are partly buried among the phospholipid molecules in the microsomal membrane. There are 3 essential phases in a hydroxylation reaction catalyzed by the mono-oxygenase complex:

1. The reduction of NADPH cytochrome *P*-450 reductase by the reduced coenzyme molecules from the solution outside the membrane.
2. The transfer of electron from NADPH cytochrome *P*-450 reductase to cytochrome *P*-450.
3. The binding of a substrate and oxygen to the active site of cytochrome *P*-450 (buried probably in the hydrophobic interior of the membrane).

The oxidation of a substrate takes place when cytochrome *P*-450 receives electrons from its reductase [19]. Microsomal phospholipids are known to be necessary for both the electron transfer and the binding of substrates for cytochrome *P*-450 but their role is not known in detail [17,20]. In our study the effects of the fluorescent probes on each of the 3 phases was determined separately.

2,6-Dichlorophenol indophenol is a water soluble substrate for NADPH cytochrome *P*-450 reductase [18] and its reduction rate is not regulated by membrane phospholipids as in the reduction of cytochrome *P*-450 [17,19]. Of the probes used, only 1,8-ANS was able slightly to lower the rate of 2,6-dichlorophenol indophenol reduction. This indicates that 1,8-ANS may interfere with the reaction between NADPH or 2,6-dichlorophenol indophenol and NADPH cytochrome *P*-450 reductase, although only weakly.

Cumene hydroperoxide delivers oxygen atoms directly to cytochrome *P*-450 and in this case no NADPH cytochrome *P*-450 reductase or NADPH is needed to maintain the hydroxylation reactions [17]. When cumene hydroperoxide was used, no inhibition of the mono-oxygenase reactions by the fluorescent probes took place. This indicates that the probes do not interfere with the binding of oxygen or substrates to the active site of cytochrome *P*-450.

The NADPH-driven mono-oxygenase reactions were inhibited effectively by 1,8-ANS and to a lesser extent by PNA. Because the probes were not found to interfere with substrate binding and reduced the direct reaction of NADPH with cytochrome *P*-450 reductase only slightly, it seems that the inhibition of the mono-oxygenase reactions by 1,8-ANS and PNA take place mainly by interfering with electron transport from NADPH cytochrome *P*-450 reductase to cytochrome *P*-450.

In earlier reports, microsomal phospholipids have been found to be important binding sites for 1,8-ANS and PNA [6–8]. 1,8-ANS has been shown to bind to the ammonium head of the phosphatidylcholine molecules [6]. Microsomal phospholipids are also

necessary for electron transport to cytochrome *P*-450 molecules [17,20].

In conclusion, 1,8-ANS and PNA inhibit microsomal drug metabolism mainly by interfering with electron transport from NADPH cytochrome *P*-450 reductase to cytochrome *P*-450. Because the inhibitory effect of 1,8-ANS was more effective than that of PNA, it is possible that the specific binding site of 1,8-ANS in the hydrophilic region of microsomal phospholipids is of importance for electron transfer.

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